Available online at www.sciencedirect.com
ScienceDirect
Journal of Microbiological Methods 72 (2008) 157–165
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates

Elke Peeters, Hans J. Nelis, Tom Coenye *

Laboratory for Pharmaceutical Microbiology, Ghent University, Harelbekestraat 72, B-9000, Ghent, Belgium

Received 26 July 2007; received in revised form 26 October 2007; accepted 13 November 2007
Available online 21 November 2007

Abstract

In the present study six assays for the quantification of biofilms formed in 96-well microtiter plates were optimised and evaluated: the crystal violet (CV) assay, the Syto9 assay, the fluorescein diacetate (FDA) assay, the resazurin assay, the XTT assay and the dimethyl methylene blue (DMMB) assay. *Pseudomonas aeruginosa*, *Burkholderia cenocepacia*, *Staphylococcus aureus*, *Propionibacterium acnes* and *Candida albicans* were used as test organisms. In general, these assays showed a broad applicability and a high repeatability for most isolates. In addition, the estimated numbers of CFUs present in the biofilms show limited variations between the different assays. Nevertheless, our data show that some assays are less suitable for the quantification of biofilms of particular isolates (e.g. the CV assay for *P. aeruginosa*).

Keywords: Biofilm quantification; Microtiter plate; Crystal violet; Resazurine; FDA; XTT

1. Introduction

Biofilms are microbial communities of surface-attached cells embedded in a self-produced extracellular polymeric matrix (Donlan and Costerton, 2002). They can cause significant problems in many areas, both in medical settings (e.g. persistent and recurrent infections, device-related infections) and in non-medical (industrial) settings (e.g. biofouling in drinking water distribution systems and food processing environments) (Flemming, 2002; Fux et al., 2005; Kumar and Anand, 1998).

Over the last decades, a broad range of model systems have been described for the *in vitro* study of biofilm formation and development (McLean et al., 2004). In most of these model systems, the quantification of biofilm biomass is done by conventional plating, which is labour-intensive and slow (Donlan and Costerton, 2002). Over the past years, several surrogate assays for biofilm quantification in microtiter plates have been described. These can be classified into biofilm biomass assays (based on the quantification of matrix and both living and dead cells), viability assays (based on the quantification of viable cells) and matrix quantification assays (based on the specific staining of matrix components).

Crystal violet (CV) staining was first described by Christensen et al. (1985) and has since then been modified to increase its accuracy and to allow biofilm biomass quantification in the entire well (Stepanovic et al., 2000). CV is a basic dye, which binds to negatively charged surface molecules and polysaccharides in the extracellular matrix (Li et al., 2003). Because cells (both living and dead), as well as matrix are stained by CV, it is poorly suited to evaluate killing of biofilm cells (Pitts et al., 2003).

The fluorogenic dye Syto9 is a nucleic acid stain, which diffuses passively through cellular membranes and binds to DNA of both viable and dead cells (Boulos et al., 1999). As DNA is also a substantial part of the extracellular matrix (Whitchurch et al., 2002), this staining will provide information on total biofilm biomass. Syto9 has previously been used in confocal laser scanning microscopy (CLSM) studies of biofilm composition and morphology (Lawrence et al., 1998; Strathmann et al., 2002). This stain has also been used for the routine quantification of bacterial and yeast biofilm biomass (Honraet et al., 2005; Honraet and Nelis, 2006).

* Corresponding author. Tel.: +32 9 2648141; fax: +32 9 2648195.
E-mail address: Tom.Coenye@Ugent.be (T. Coenye).

0167-7012/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2007.11.010

To discriminate between living and dead cells, quantification techniques based on the metabolic activity of viable cells are available. Various viability stains involve the use of tetrazolium salts, including 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) (Gabrielson et al., 2002; McCluskey et al., 2005). The XTT assay is based on the reduction of the XTT dye to a water-soluble formazan (Roehm et al., 1991). The absorbance of the cell supernatant is proportional to the number of metabolically active microbial cells. The XTT assay has been used extensively for the quantification of viable cells in planktonic cultures (Gabrielson et al., 2002) and for the quantification of bacterial (Berit et al., 2002; Pettit et al., 2005) and yeast biofilms (Honraet et al., 2005). Despite its popularity, problems regarding intra- and interspecies variability have been reported (Kuhn et al., 2003; Honraet et al., 2005).

Another viability assay is based on the reduction of resazurin by metabolically active cells. This is a blue compound that can be reduced to pink resorufin, which is fluorescent (O'Brien et al., 2000). Although this dye, also known as Alamar Blue, is mainly used in viability assays for mammalian cells, it has also been applied extensively in susceptibility testing of fungi, *Mycobacterium tuberculosis*, *Staphylococcus epidermidis* and *Streptococcus mutans* (Carrillo-Muñoz et al., 2006; Palomino et al., 2002; Pettit et al., 2005; Wei et al., 2006).

Viable microbial cells are also capable of converting non-coloured, non-fluorescent fluorescein diacetate (FDA) into yellow, highly fluorescent fluorescein by non-specific intra- and extracellular esterases. FDA has been used for measuring total microbial activity in soil and litter (Adam and Duncan, 2001; Schnürer and Rosswall, 1982; Taylor et al., 2001), as well as for the quantification of biofilm biomass (Honraet et al., 2005; Prieto et al., 2004).

The quantification of the biofilm matrix, an essential component of the biofilm, is also of interest. The dye 1,9-dimethyl methylene blue (DMMB), originally applied for quantification of sulphated glycosaminoglycans in chondrocyt cultures (Enobakhare et al., 1996), has also been used to quantify *Staphylococcus aureus* biofilm matrices (Toté et al., 2007). DMMB forms an insoluble complexation product with sulphated polysaccharides in the biofilm matrix. The amount of dye released by adding a decomplexation solution is spectrophotometrically measured and reflects the amount of sulphated polysaccharides present in the biofilm matrix (Barbosa et al., 2003).

In the present paper various methods for the quantification of microorganisms were compared. To this end, these methods were optimised and subsequently their applicability and repeatability for the quantification of bacterial and yeast biofilms formed in a microtiter plate were evaluated.

2. Materials and methods

2.1. Strains and culture conditions

Burkholderia cenocepacia strains LMG 18828 and LMG 16656, *S. aureus* strains ATCC 6538 and ATCC 700699 and *Pseudomonas aeruginosa* strains PAO1 and ATCC 9027 were cultured aerobically on Nutrient Agar (Oxoid, Hampshire, UK) or in Tryptone Soya Broth (Oxoid) at 37 °C. *Candida albicans* SC 5314 and ATCC 10231 were grown aerobically on Sabouraud Dextrose Agar (SDA; Oxoid) or in Sabouraud Liquid Medium (Oxoid) at 37 °C. *Propionibacterium acnes* LMG 16711 was grown anaerobically (Anaerocult A mini, Merck, Darmstadt, Germany) on Reinforced Clostridial Agar (RCA; Oxoid) or in Liquid Reinforced Clostridium Medium (RCM; Oxoid) at 37 °C.

2.2. Biofilm formation

Starting from an overnight liquid culture (a 60–70 h old culture for *P. acnes*), dilutions containing approximately 10^8 CFUs/ml (bacteria) or 10^7 CFUs/ml (yeasts), were made. For each biofilm experiment, 24 wells of a round-bottomed polystyrene 96-well microtiter plate (TPP, Trasadingen, Switzerland) were inoculated with 100 µl of these dilutions and 24 control wells were filled with sterile medium. Following 4 h of adhesion, the supernatant (containing non-adhered cells) was removed from each well and plates were rinsed using 100 µl physiological saline (PS). Subsequently, 100 µl of fresh medium was added to each well and the plates were further incubated for 24 h.

After 4 h adhesion and 24 h biofilm formation, the supernatant was again removed and the wells were rinsed with 100 µl of MOPS buffer (for the FDA assay) or PS (for all other assays). One litre MOPS buffer contains 20.9 g MOPS (Sigma, St. Louis, MO, USA) and 5.6 g NaCl (Novolab, Geraardsbergen, Belgium); pH 7.00. All assays were repeated at least six times per strain.

2.3. Measurement of fluorescence and absorbance signals

Fluorescence and absorbance signals were measured using a multilabel microtiter plate reader (Wallac Victor^2; Perkin Elmer Life and Analytical Sciences, Boston, MA, USA) equipped with filters with a band pass ranging from 14 to 40 nm.

2.4. Biofilm quantification

2.4.1. Crystal violet assay (CV assay)

For fixation of the biofilms, 100 µl 99% methanol was added (15 min), after which supernatants were removed and the plates were air-dried. Then, 100 µl of a CV solution was added to all wells. After 20 min, the excess CV was removed by washing the plates under running tap water. Finally, bound CV was released by adding 150 µl of 33% acetic acid (Sigma). The absorbance was measured at 590 nm. All steps were carried out at room temperature.

In order to determine the optimal concentration of CV for each isolate, several twofold dilutions of a CV stock solution (0.5%; Pro-lab Diagnostics, Richmond Hill, ON, Canada) in MilliQ water (Millipore, Billerica, MA, USA) were tested prior to the actual analysis.

2.4.2. Syto9 assay

One hundred µl of PS was added to each well, followed by the addition of 100 µl Syto9 working solution, prepared by diluting